Unpeaceful roles of mutant PAX proteins in cancer

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Review

Unpeaceful roles of mutant PAX proteins in cancer

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ABSTRACT

PAX transcription factors are key players in the development of different tissues and organs. At the cellular level they are involved in regulating lineage commitment and differentiation. Interference with these tightly regulated functions of PAX proteins is associated with developmental abnormalities and tumorigenesis of several types of cancer. As a result of aberrant PAX protein activity, either by gain- or loss of function mechanisms, affected cells are kept in a proliferative state by blocking their terminal differentiation. PAX proteins with a gain-of-function role in cancer are active in the proliferative state of cells and have to be downregulated before they can complete the differentiation process. Such PAX proteins are usually activated in malignancies by chromosomal translocations generating fusions with strong transcriptional activators. PAX proteins with tumor suppressor activity are actively driving the differentiation process and are necessary for the exit from the proliferative state. In cancer, a diverse set of mutational mechanisms is involved in reducing their activity.

Here, we discuss the characteristics of mutant PAX proteins in different types of cancer including alveolar rhabdomyosarcoma, biphenotypic sinonasal sarcoma, thyroid cancer and leukemia, with special focus on their role in interference with normal differentiation pathways of the cell lineage involved.

Contents

1. Introduction

PAX proteins play important roles during embryogenesis and are crucial for the development of various tissues and organs. Hence, aberrant activity of different PAX proteins is associated with several developmental defects. In the adult organism, PAX proteins remain expressed mainly in stem/progenitor cell populations but also in some mature cells and are functionally involved in tissue regeneration and maintenance [1]. At the cellular level, PAX proteins are important regulators of lineage commitment and differentiation. This functional role as molecular switches between proliferative and differentiated states of cells characterizes PAX proteins as potential tumorigenic proteins. Indeed, in the last two

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decades it became evident that aberrant PAX protein activity is causally involved in tumorigenesis of different malignancies. Most of the nine PAX protein family members have been associated with a role in cancer [2]. One type of mechanism by which PAX proteins contribute to tumorigenesis is the expression of inappropriate levels of wildtype proteins, which plays an important role in numerous tumor types. These quantitative disturbances are beyond the scope of this review and the reader is referred to other reviews in the field [2–4]. Here, we specifically focus on genetic aberrations of PAX proteins involved in tumorigenesis and discuss a model that correlates aberrant PAX protein activity with permissive cellular states present in affected cell lineages. In this context both gain and loss of function roles of PAX proteins have been described. The prime examples for this yin and yang function of PAX proteins are PAX3/7 in alveolar rhabdomyosarcoma and biphenotypic sinonasal sarcoma, PAX8 in thyroid cancer and PAX5 in B-cell acute lymphoblastic leukemia, which we will discuss in detail.

2. Gain of function roles of PAX proteins in cancer

2.1. PAX3 and PAX7

2.1.1. PAX3 and PAX7 in normal development

PAX3 and PAX7 are important regulators of neural tube, neural crest and skeletal muscle development. Based on the characteristics of the tumors expressing aberrant PAX3/7 proteins, of special interest here are the roles of PAX3 and PAX7 in the myogenic and neural crest-derived lineages.

During embryonic development the process of myogenesis starts with embryonic progenitors in the dermomyotomal part of the somites (for review and details see [5]). These cells migrate to reach the final anatomic location where they terminally differentiate or form a heterogeneous pool of stem and committed cells called satellite cells. Both PAX3 and PAX7 are expressed in the dermomyotome, however, they fulfill different functions during myogenesis. PAX3 but not PAX7 is expressed in long-range migrating cells which form the initial limb musculature and PAX3 deficient mice do not develop limb or diaphragm muscles [6–8]. PAX7 appears to be dispensable for embryonic muscle development and instead is responsible for renewal and maintenance of the satellite cell pool [9,10].

At the cellular level, embryonic progenitors give rise to activated committed cells (myoblasts). The subsequent differentiation process then leads to myocytes which finally fuse to form multinucleated myotubes (Fig. 1) [11]. A genetic hierarchy regulates these different steps, initiated by the upstream regulators sine oculis-related homeobox 1 and 4 (Six 1 and −4), followed by activation of PAX3 and PAX7 which direct expression of the downstream myogenic regulatory factor (MRF) family (Myf5, MyoD, Myogenin and Myf6 (Mrf4)). PAX3 and PAX7 are specifically active during early stages of lineage commitment and are downregulated to allow terminal differentiation into muscle cells (Fig. 1) [12,13]. Maintenance of PAX3 and PAX7 expression prevents differentiation of myoblasts and satellite cells, respectively [14,15].

PAX3 also has a prominent role in the neural crest lineage (for review see [16]). Neural crest cells give rise to a wide range of derivatives including cells of the peripheral nervous system (PNS), melanocytes, endocrine cells and mesenchymal cells able to differentiate into connective, adipose, bone, cartilage tissues and pericytes [17]. In the mouse, PAX3 is first detected at E8.5 in the dorsal neuroepithelum and later, in neural crest cells of the developing PNS, the neural crest-derived craniofacial mesenchyme and the migratory cardiac neural crest cells [18]. Similar to the myogenic

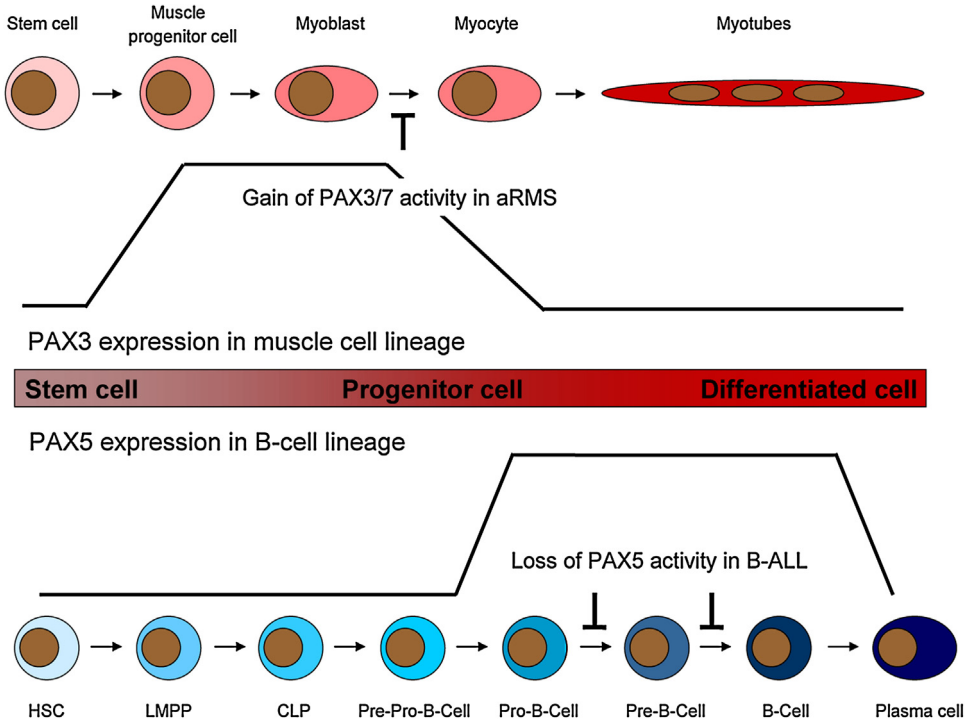

Fig. 1. Expression of PAX3/7 during skeletal muscle development and PAX5 during B-cell development and stages of gain and loss of their activities in associated tumors. In the myogenic lineage, PAX3/7 expression is induced in muscle progenitor cells and is involved in commitment to the myogenic lineage. Before terminal differentiation is executed, PAX3/7 is downregulated. Aberrant activity of PAX3/7-FOXO1 blocks differentiation and promotes tumorigenesis. During B cell development, PAX5 expression starts at the pro-B cell stage and is involved in commitment to the B cell lineage. It is stably expressed throughout B cell development and downregulated at the initiation of plasma cell differentiation. Loss of PAX5 activity blocks differentiation and acts as cooperative hit in tumorigenesis of B-ALL. HSC, hematopoietic stem cells, LMPP, lymphoid-primed multipotent progenitors, CLP, common lymphoid progenitors.

Table 1
PAX fusion proteins in cancer.

Tumor type	*Fusion protein*	*Frequency [%]*	*Function of partner protein*	*Effects beyond PAX*	*Ref.*
Alveolar rhabdomyosarcoma	PAX3-FOXO1	60	Transcription factor		[122]
	PAX7-FOXO1	20	Transcription factor		[123]
	PAX3-FOXO4	Single case	Transcription factor		[22]
	PAX3-NCOA1	Recurrent	Nuclear receptor coactivator		[24,25]
	PAX3-NCOA2	Recurrent	Nuclear receptor coactivator		[24]
	PAX3-INO80D	Single case	Regulatory component of INO80 chromatin remodeling complex		[23]
Biphenotypic sinonasal sarcoma	PAX3-MAML3	79	Transcriptional coactivator for Notch intracellular domain		[78]
B-ALL	PAX5-ETV6	Up to 1	Transcription factor		[100,106,124,125]
	PAX5-PML	Recurrent	Scaffolding protein for PML nuclear bodies	Inhibition of PML functions	[111,126]
	PAX5-FOXP1	Recurrent	Transcriptional repressor		[100,106]
	PAX5-ZNF521	Single case	Transcription factor		[100]
	PAX5-ELN	Recurrent	Extracellular matrix protein		[106,107]
	PAX5-AUTS2	Recurrent	not known		[106,127]
	PAX5-C20orf112	Recurrent	not known		[105,124,127]
	PAX5-JAK2	Recurrent	Non-receptor tyrosine kinase	Activation of JAK-STAT pathway	[105,106,109]
	PAX5-*BRD1*	Single case	Component of the MOZ/MORFH3 acetyltransferase complex		[105]
	PAX5-POM121	Recurrent	Component of the nuclear pore complex		[105,106]
	PAX5-HIPK1	Single case	Serine/threonine kinase – corepressor for homeodomain TFs		[105]
	PAX5-DACH1	Single case	Transcription factor		[105]
	PAX5-*LOC392027*	Single case	Pseudogene		[124]
	PAX5-*SLCO1B3*	Single case	Organic anion transporter		[124]
	PAX5-*ASXL1*	Recurrent	Nuclear receptor coactivator		[124]
	PAX5-*KIF3B*	Single case	Component of kinesin motor complex		[124]
	PAX5-DACH2	Single case	Transcription factor		[106]
	PAX5-NCoR1	Single case	Nuclear receptor corepressor		[106]
	PAX5-GOLGA6	Single case	not known		[106]
	PAX5-*TAOK1*	Single case	Serine/threonine kinase		[106]
	PAX5-MLLT3	Single case	Component of the super elongation complex		[128]
	PAX5-CHFR	Single case	E3 ubiquitin-protein ligase		[128]
	PAX5-SOX5	Single case	Transcription factor		[128]
	PAX5-POM121C	Single case	Component of the nuclear pore complex		[128]
Follicular thyroid carcinoma	PAX8-PPARγ	30–35	Nuclear receptor	Deregulation of PPARγ target genes	[129]
Follicular-variant papillary thyroid carcinoma	PAX8-PPARγ	Recurrent	Nuclear receptor	Deregulation of PPARγ target genes	[83]

PAX5 fusions with fusion partner in reverse orientation or out of frame are depicted in italic.

lineage, PAX3 expression is generally downregulated upon differentiation and PAX3-deregulation in cranial neural crest cells results in developmental defects in the head region, suggesting that also in neural crest cells PAX3 acts as upstream regulator of stemness and differentiation [19]. In the mouse, PAX7 is also expressed in the cranial neural crest, however its role in humans is less clear and PAX7 was not detected in the neural crest [20]. However, both PAX3 and PAX7 expression was noted in a range of neuroectodermal tumors such neurofibroma (PAX3), malignant nerve sheet tumor (PAX3 and PAX7), melanoma (PAX3), Ewing sarcoma (PAX3 and PAX7), medulloblastoma (PAX3) and primitive neuroectodermal tumor (PAX3) [20].

2.1.2. *PAX3 and PAX7 in alveolar rhabdomyosarcoma*

Chromosomal translocations affecting PAX3 or PAX7 are associated with development of alveolar rhabdomyosarcoma (aRMS). ARMS represents a histological subgroup of rhabdomyosarcoma, the most common soft tissue sarcoma in children. In 80% of aRMS cases the *PAX3* or the *PAX7* gene are rearranged. In about 60% of these a chromosomal translocation t(2;13)(q35;q14) generates the fusion protein PAX3-FOXO1, while a similar translocation t(1;13)(p36;q14) associated with PAX7-FOXO1 is found in about 20% of cases [21]. In a small number of patients alternative fusions including PAX3-FOXO4, PAX3-NCOA1, PAX3-NCOA2 and PAX3-INO80D have been found [22–25] (Table 1). All fusion partners have a role in transcriptional regulation. FOXO1 and −4 are transcription factors, NCOA1 and −2 are nuclear receptor coactivators and INO80D is a subunit of the INO80 chromatin remodeling complex. In the fusion proteins, the DNA-binding domains of the PAX protein are linked to the transcriptional transactivation domain of the fusion partner. Hence, the fusion proteins act as aberrant transcription factors at PAX3 or PAX7 target genes. High levels of fusion protein activity appear to be relevant for aRMS development, with basal transcriptional activity of fusion proteins being much higher than the one of the wildtype PAX proteins [25,26]. Additional mechanisms further contribute to boost their activity in aRMS, including gene amplification in the majority of PAX7-FOXO1 cases [27], copy-number independent increase of transcriptional activity in case of PAX3-FOXO1 [28] and increased protein stability [29,30]. Furthermore, negative regulation by phosphorylation-induced sequestering of proteins into the cytoplasm, a mechanism by which different kinases including Akt

negatively affect the activity of all wildtype FOXO family members [31–33], is not active in the context of the fusion protein. Probably as a result of the dominating effect of the nuclear localization signal present in the PAX N-terminus, the fusion proteins are permanently localized in the nucleus [31]. Akt-mediated phosphorylation might have a direct effect on PAX3-FOXO1 transcriptional activity depending on its strength, with low Akt signaling associated with elevated PAX3-FOXO1 transcriptional activity and hyperactivated Akt associated with repressed PAX3-FOXO1 activity [34]. These observations strongly suggest that the fusion proteins act by deregulation of a PAX3 or PAX7 target gene signature. The PAX3/7-FOXO1 dependent gene signature has been studied in the past decade by several groups and contains several hundred mostly upregulated genes [25,35–38], however, the relevance of the individual genes for aRMS tumorigenesis is still only poorly understood. The translocations are the main recurrent genetic aberrations found in aRMS, which otherwise have a very low somatic mutation burden, and are thought to be the first genetic hit [23,39]. In a recent genome sequencing study of a large cohort of aRMS tumors one case was found having no protein-coding somatic alterations apart from the PAX3-FOXO1 translocation and a copy number neutral LOH on chromosome 11p [23]. Taken together, this suggests that the PAX fusion proteins act as the major oncogenic drivers in aRMS.

The cell (lineage) of origin of aRMS is still debated. Most frequently, aRMS tumors are found in the extremities [40], but in general they can appear throughout the body [40] even at sites unexpected for a soft tissue sarcoma such as the bone marrow [41–43]. Hence no clear association between primary tumor site and tissue of origin exists. However, RMS tumors in general are characterized by expression of myogenic differentiation markers, which is the basis for their naming. On the cytological level this includes presence of (rare) cross-striated and multinucleated cells [44] and on the molecular level expression of a range of skeletal muscle specific marker proteins including skeletal muscle actin, myosin, myoglobin, Z-band protein, MYOD and myogenin [45–47]. These features are reminiscent for an interrupted skeletal muscle differentiation and have led to the hypothesis that RMS originates from the myogenic lineage. Uncontrolled high activity of the fusion protein in a committed cell of the myogenic lineage might prevent these cells from undergoing terminal differentiation and trap them in a proliferative state. Indeed, different lines of evidence support this hypothesis. First, silencing of the fusion protein by siRNA in *in vitro* cultured aRMS cell lines induces, apart from cell death, a strong upregulation of a range of muscle markers including troponin C, troponin I, crystalline aB and myosin light and heavy chain [38], suggesting that downregulation of the fusion protein allows the cells to further execute the myogenic differentiation program. Second, in *in vitro* transformation experiments it was shown that expression of PAX3-FOXO1 in combination with hTERT and NMyc can convert primary human myoblasts into tumorigenic cells producing aRMS like tumors in mice [48]. Finally, conditional expression of PAX3-FOXO1 during different stages of the myogenic lineage in combination with loss of p53 or Ink4a, induces formation of tumors in mice [49,50]. Most susceptible for formation of tumors resembling human aRMS was found to be the fetal myoblast stage (Myf6 lineage) [50]. Interestingly, normally Myf6 is expressed in terminally differentiating myoblasts at stages where PAX3 expression is already shut down, raising the provocative possibility that aRMS tumors originate from terminally-differentiating, Myf6-expressing myofibres [49]. In accordance with this finding is data from a Drosophila model, where expression of PAX7-FOXO1 in differentiated myofibers can drive the generation of nucleated cells [51]. Together, these data are consistent with a model in which the fusion protein would be able to induce dedifferentiation toward a stem cell state. Interestingly however, during embryogenesis Myf6 shows a biphasic expression pattern with a first transient activation in myotomes during somitogenesis and a second wave of expression in fetal skeletal muscle [52]. Myf6 expression in some cells of the ventral thoracic somites is earlier than during myogenesis and precedes expression of MyoD [53]. Such cells might represent an alternative pool for tumor development upon PAX3-FOXO1 expression, without the necessity for reversal of differentiation [54].

A central mechanism by which the fusion protein might block the differentiation process of the myogenic lineage might be interference with the activity of the MRFs, especially MyoD (for review see [55]). PAX3-FOXO1 interferes with the activity of MyoD by disturbing its capability to remodel the chromatin at its target gene sites necessary for transactivation [56]. Interestingly, integrative analysis of mutation and gene expression data from aRMS and embryonal RMS (eRMS), a related tumor also potentially originating from the myogenic lineage and similarly displaying a block in the myogenic differentiation program, revealed a significant overlap between PAX3(7)-FOXO1 target genes involved in growth factor signaling in aRMS and genes mutated in eRMS. This suggests that different mechanisms, having similar effects on differentiation, are active in these two related tumor types with myogenic features [23].

Importantly however, both *in vitro* and *in vivo* expression of PAX3-FOXO1 alone was not sufficient to promote transformation and tumorigenesis (tumor incidence in wildtype mice expressing PAX3-FOXO1 was only 1/228). Other models confirmed this finding [57]. Hence, the fusion protein might not be the only genetic event necessary for rhabdomyosarcomagenesis. However, loss of p53 or Ink4a, which were used in the mouse models, are rarely found in human aRMS [23,39], suggesting that these models might also have caveats to consider. On the other hand it can not be excluded that the myogenic expression pattern found in aRMS is rather a reflection of the activity of the fusion proteins themselves than of the original transcriptome of the cell of origin. Forced expression of all members of the MRF familiy in cells of non-myogenic origin is able to convert these cells into muscle cells [58–61]. Also PAX3 has the ability to induce myogenic commitment, albeit only in specific cell types, mainly of mesenchymal origin [62]. Along these lines, different alternative cells of origins have been discussed. These include pericytes/mesangioblasts [50] and mesenchymal stem cells (MSCs) [63]. The latter hypothesis is based on the fact that in some aRMS patients, both of the PAX3-FOXO1 and the PAX7-FOXO1 type, tumor cells have been found exclusively in the bone marrow, without a detectable primary tumor present elsewhere [41–43,64]. This suggests that the bone marrow might not be a metatstatic but in fact the primary site of tumor development. MSCs are present in most postnatal tissues including the bone marrow and differentiate into tissues of mesodermal origin such as adipocytes, osteoblasts or skeletal myocytes [65]. Similar to the experiments performed with human myoblasts, sole expression of PAX3-FOXO1 in mouse MSCs did not transform these cells to a state capable to form xenograft tumors in mice. However, in combination with cooperative events such as presence of dominant-negative p53, xenograft tumors with an aRMS like gene expression pattern did grow in mice [66].

The potential cell of origin might be an even less committed cell like a neural crest cell. Interestingly, aRMS cells express a large signature of neurogenesis-associated genes, at least some of them target genes of PAX-FOXO1 [67], potentially reflecting the multipotency of the cell of origin. Furthermore, AP2β, a marker for neural crest cells [68], is also highly expressed and used as marker for aRMS, allowing discrimination from eRMS [69,70]. Potentially, even more than one cell of origin could be involved. In this respect, it is also not known whether PAX3-FOXO1 and PAX7-FOXO1 positive aRMS originate from the same cell of origin. On the one hand PAX3 and PAX7 have a very high sequence identity of 93 and 97% in the paired box and homeobox domains, respectively, and it has

been suggested that they originated during evolution by duplication of a common ancestral gene [71]. Indeed, replacement of PAX3 by a PAX7 knock-in was shown to restore most of the functions of PAX3 during embryonic development [72]. Therefore, it is thought that they bind to a similar set of target genes. Comparison of PAX3-FOXO1 and PAX7-FOXO1 gene expression data in aRMS confirmed that discriminating gene sets are small and not strictly correlated to the two fusion genes [67]. On the other hand, PAX3 and PAX7 have distinct functions during development and also in aRMS differences between PAX3-FOXO1 and PAX7-FOXO1 cases have been described. Most importantly, the clinical outcome of patients with PAX7-FOXO1 has been claimed by several studies to be significantly better when compared to PAX3-FOXO1 cases with 4–5 year overall survival rates of 74–92% (75% in case of metastatic tumors) of patients with PAX7-FOXO1 tumors *versus* 35–64% (8% in case of metastatic tumors) of patients with PAX3-FOXO1 tumors [21,67,73,74], albeit in a European cohort such differences were not detected [75]. Accordingly, compared to PAX7-FOXO1 positive tumors, PAX3-FOXO1 positive aRMS are more often associated with known adverse clinical risk-factors such as patient age higher than 10 years, tumor invasiveness and staging into poorer risk groups [21,75,76]. Additionally, some biological features of tumor cells have been found to differ between PAX3-FOXO1 and PAX7-FOXO1 aRMS tumors such as expression of markers for active cell cycle and apoptosis [77].

Irrespective of the cell of origin however, it seems obvious that PAX3 and PAX7 act early in the involved cell lineage(s) and that uncontrolled upregulation of their activity in the context of the fusion proteins blocks terminal differentiation of these cells.

2.1.3. PAX3 in biphenotypic sinonasal sarcoma

Recently, rearrangement of the *PAX3* locus has also been found in 96% of biphenotypic sinonasal sarcoma (SNS) and in 79% of the cases a fusion of *PAX3* with *MAML3* was involved [78]. MAML3 is one of three structurally divergent Mastermind (MAM) isoforms (MAML1, MAML2 and MAML3), which act as transcriptional coactivators of the Notch intracellular domain [79]. Only MAML1 and MAML3 are essential for Notch signaling *in vivo* and their combined knock-out is embryonically lethal in the mouse [80]. Similar to the fusion proteins found in aRMS, also in PAX3-MAML3 the DNA binding part of PAX3 is fused to the transactivation domain of the fusion partner. Accordingly, also PAX3-MAML3 has a transcriptional activity which is much higher than the one of wildtype PAX3.

SNS is a distinct spindle cell sarcoma found in the sinonasal region of mainly middle aged adults, with women more frequently affected than men [81]. Also SNS show both neural and myogenic features [81] and expression profiling confirmed that the tumors express genes involved in neuroectodermal and myogenic differentiation [78]. However, transcriptome analysis demonstrated that SNS clearly separate from aRMS tumors, highlighting that sarcoma characteristics are dependent on different cooperative parameters including cell of origin and characteristics of the fusion protein. The cell of origin is not yet known for this tumor entity. Nevertheless, the characteristics of SNS reflect the developmental roles of PAX3 in the differentiation and migration of neural crest cells and other cells of ectodermal and mesodermal lineage. Hence, similar to the fusion proteins in aRMS, an important aspect of the oncogenic activity of PAX3-MAML3 might be blocking of differentiation and trapping these cells in a proliferative precursor state.

2.2. PAX8 in thyroid cancer

While PAX8 protein is also expressed in the developing brain and kidney, its major role lies in the development of the thyroid gland. Hence, the only abnormality in *PAX8* knock-out mice is the absence of a thyroid gland and in humans, *PAX8* mutations are the cause of congenital hypothyroidism [82]. The *PAX8* gene is rearranged in about 30–35% of follicular thyroid carcinomas (FTC), some follicular-variant papillary thyroid carcinoma and occasionally in follicular adenomas [83,84]. In these tumors translocation t(2;3)(q13;p25) leads to fusion with the *PPARG* gene. The corresponding protein PPAR-γ is a transcription factor of the nuclear receptor family acting as master regulator of adipogenesis. Endogenous ligands include fatty acids and eicosanoids [85]. In the PAX8-PPAR-γ fusion a truncated part of PAX8 containing the DNA binding domain but lacking part of the transactivation domain is fused to full length PPAR-γ1. The fusion protein therefore has the potential to affect both PAX8 and PPAR-γ target genes. A range of *in vitro* studies using cultured thyrocytes demonstrated that the fusion protein induces cell proliferation, blocks cell death and permits anchorage-independent growth, typical for an oncogenic behavior [86–88]. Again, in transgenic mice PAX8-PPAR-γ alone is not sufficient to induce tumor development, however expression in thyroid cells in combination with homozygous deletion of *Pten* induced formation of thyroid carcinoma [89]. The functional contributions of the two partners in the fusion are only partially understood, however effects on expression of target genes of both fusion partners might play a role. PAX8 target genes are variably stimulated or repressed in PAX8-PPAR-γ transfected cells [86]. Similarly, also PPAR-γ target genes are either stimulated or repressed. Since it was shown in *in vitro* studies that the fusion protein can inhibit some properties of wildtype PPAR-γ it was postulated that it might interfere with a potential tumor suppressive function of PPAR-γ. However, in gene expression studies of FTC with and without PAX8-PPAR-γ it was found that PPAR-γ target gene signatures known from adipocytes are upregulated in the PAX8-PPAR-γ positive tumors [90,91]. To make it even more complex, treating the transgenic mouse model with a PPAR-γ agonist had a strong antitumor effect despite induction of many PPAR-γ target genes in the tumor cells [89].

Hence, the contribution of hijacked PAX8 and PPAR-γ functions to tumorigenesis and especially their involvement in block of differentiation of thyroid cancer cells needs further clarification.

3. Loss of function roles of PAX proteins in cancer

3.1. PAX5

3.1.1. PAX5 in normal B-cell development

In contrast to the oncogenic activities of the mutant forms of PAX3, PAX7 and PAX8 described above, PAX5 acts as tumor suppressor in the haematopoietic lineage. Reduction of its activity is causally involved in tumorigenesis of some B-cell precursor acute lymphoblastic leukemia (B-ALL).

PAX5 plays an important role in normal B-cell development [92]. B-cell development is a stepwise process that is initiated in the bone marrow with the differentiation of hematopoietic stem cells (HSCs) into lymphoid-primed multipotent progenitors (LMPPs) followed by common lymphoid progenitors (CLPs) (Fig. 1). CLPs then give rise to pre-pro-B cells, which subsequently differentiate into pro-B cells, where commitment to the B cell lineage occurs [93] and *Igh* recombination takes place. Successful *Igh* recombination leads to expression of IgH on the cell surface, which together with surrogate light chain and accessory signaling molecules (Igα, Igβ) forms the pre-B cell receptor (pre-BCR) and characterizes the large pre-B cell state. Activation of the pre-BCR induces a burst of proliferation [94], activates recombination of the *Igl* locus and triggers differentiation into the small pre-B cell stage. Successful rearrangement of the *Igl* locus then leads to the expression of BCR and progression to the immature B cell stage. These cells exit the bone marrow and complete their development to the mature B cells in the periphery.

At the molecular level, these hierarchical cellular changes are controlled by a set of transcription factors that act in a complex network to control the respective processes. PAX5 plays a central role in this network. PAX5 is not required for the initial stages of B cell development but its expression starts at the pro-B cell stage until it is downregulated during antigen driven differentiation of mature B cells into immunglobulin secreting plasma cells [95]. PAX5 is necessary for the execution of the differentiation program in stages following the pro-B cell state, based on its ability to repress B cell lineage inappropriate genes. Consequently, in *PAX5*-deficient mice B-cell development is arrested at the pro-B cell stage [96]. In lieu thereof, such *PAX5*-negative pro B-cells are able to differentiate into different other hematopoietic cell types such as T cells or myeloid cells [97]. The B cell lineage is characterized by some plasticity since deletion of *PAX5* even in mature B cells results in dedifferentiation to pro-B cells and lymphomagenesis [98].

3.1.2. *PAX5 in leukemia*

PAX5 is mutated in about 40% of both childhood and adult precursor B-cell acute lymphoblastic leukemia (B-ALL) and in some cases of chronic myelogenous leukemia (CML) at the time of progression to acute leukemia blast crisis by a diverse set of mechanisms [99–102]. Furthermore, germline hypomorphic mutations in *PAX5* are associated with B-ALL susceptibility, albeit additional loss of the wildtype allele was always seen in such B-ALL cases [103]. The most frequent aberrations found in about 30% of cases are monoallelic deletions affecting the entire or part of the *PAX5* gene, leading to reduction of the expressed PAX5 protein [100]. Interestingly, during the earliest phase of B cell commitment only a single allele of *PAX5* is transcribed, before expression switches to a bi-allelic mode when B cells begin to differentiate [104]. It was speculated that loss of one allele therefore might interfere with the ability of the cell to increase expression levels by inducing bi-allelic expression [100]. A second type of mutation found in about 5–7% of cases are point mutations that affect the DNA-binding or transcriptional regulatory domains of the protein which result in lost or altered DNA-binding or transcriptional activity of the protein. Finally, in 2–3% of cases translocations involving the *PAX5* gene have been found generating different chimaeric PAX5-fusion proteins [100,101,105]. *PAX5* fusion partners are a very heterogeneous group of genes including transcription factors, structural proteins and kinases (Table 1). In some cases out of-frame fusions, fusion with a gene in opposite direction and fusion with non-coding sequences have been described which can lead to generation of truncated proteins as a result of premature stop codons [106]. Importantly however, and similar to the structure of PAX fusion proteins found in other cancers, all described PAX5 fusion proteins retain the paired domain and most also octapeptide and nuclear localization signal of PAX5, suggesting that they bind to DNA and negatively influence the activity of wildtype PAX5 on target gene expression. Indeed, competitive reporter assays performed with some of the fusion proteins including PAX5-ELN, PAX5-ETV6 or PAX5-FOXP1 demonstrated that these fusion proteins act as competitive inhibitors of wildtype PAX5 [100,107]. Interestingly however, some of the fusion proteins were recently found to retain some transcriptional activity, inducing transcription at least from the PAX5-responsive *CD79A* promoter in a reporter assay [108]. Furthermore, for PAX5-JAK2 [109] and for PAX5-ETV6 ectopically expressed in mouse pre-B cells [110], it was claimed that the fusion not simply antagonizes PAX5 function but influences expression of PAX5 targets in a more complex manner, potentially also activating some PAX5 target genes in the endogenous context. Finally, the PAX5 fusion partner might also exert additional effects beyond the PAX5 target gene signature that play a role in tumorigenesis (Table 1). In case of PAX5-PML the fusion protein has been shown to inhibit sumoylation of wildtype PML, and thereby interferes with PML nuclear body formation, which is associated with increased resistance to apoptosis [111]. In case of the PAX5-JAK2 fusion, the JAK2 part is able to activate the JAK-STAT pathway, leading to induction of a STAT expression signature, suggesting that JAK2 inhibitors might be a therapeutic option for tumors with this type of fusion protein [109]. Altogether, this suggests that the different PAX5 fusion proteins might exert distinct effects in B-ALL. Their mode of action might go beyond pure reduction of PAX5 activity as it is the case in the tumors with monoallelic loss of the *PAX5* gene. In accordance with such a model, most of the cases with a monoallelic deletion of *PAX5* display a complex karyotype and are associated with some of the classic recurrent translocations found in ALL such as *BCR-ABL* (frequency among B-ALL 2–4%), *TCF3-PBX* (2–6%), *ETV6-RUNX1* (15–25%) and *IGH* translocation (2–3%), however not with *MLL* rearrangements (6%), suggesting that the *PAX5* deletion is a secondary event in some of these tumors and appears rather late in the oncogenic process, potentially in subclones of the tumor [102,106,112,113]. In contrast, in cases with PAX5 fusion proteins or PAX5 truncated proteins, the *PAX5* rearrangement was mostly the sole gross chromosomal abnormality, suggesting that such an event occurs earlier in the oncogenic process [106], similar to *PAX3/7* rearrangements.

Overall, the data strongly suggests that PAX5 acts as tumor suppressor in B-ALL. It supports a model where haploinsufficient reduction of PAX5 activity in an early B-cell stage disables further B cell differentiation, which then cooperates with other aberrations in development of B-ALL. This conclusion is further supported by the fact that apart from *PAX5* other genes coding for transcriptional regulators of lymphoid development including *IKZF1*, *IKZF3*, *LEF1*, *TCF3*, and *EBF1*, the latter two direct regulators of PAX5 expression, are also frequently affected by loss-of-function mutations or deletions in B-ALL (together with *PAX5* in two-thirds of all cases) suggesting that block of B-cells in certain precursor states is a general mechanism relevant for tumorigenic transformation of cells in the B-cell lineage [100].

However, details of the tumorigenic process are not clear yet. Pro-B cells have the capacity for self-renewal and unlimited *in vitro* proliferation when cultured on stroma in presence of IL-7 [114,115]. In accordance, the most common subtype of B-ALL with 60–65% of cases is early pre-B-ALL (pro-B), suggesting that a block at this proliferative stage often contributes to human B-ALL leukemogenesis [116]. However, in B-ALL also other stages of B-cell development are represented, with the pre-B form of ALL accounting for 20–25% and mature B-cell leukemia accounting for 2–3% of ALL. *PAX5* aberrations have been found in all these stages [102]. This suggests that the situation in the context human B-ALL is more complex and not one-to-one comparable with mouse knock-out models. Such differences might also explain why *PAX5* heterozygous mice have a normal B-cell development [117], while in development of B-ALL loss of approximately half of the PAX5 activity blocks differentiation of these cells [118]. However, since loss of *PAX5* is only one among several tumorigenic events necessary for development of B-ALL, reduction of PAX5 activity might affect the cell differentiation more efficiently in an aberrant state.

Different mouse models have been generated to study the contribution of *PAX5* loss to B-ALL leukemogenesis. Similar to the other PAX proteins described here, heterozygosity of *PAX5* alone is not sufficient to induce tumor formation in mice [119]. However, in a transgenic B-ALL model driven by hematopoietic expression of constitutively active STAT5, which alone induces B-ALL with a relatively long latency and low penetrance of 1–2% [120], loss of one allele of *PAX5* dramatically accelerated leukemia formation in 100% of the animals [119]. Importantly, and comparable to human tumors, the wildtype allele of *PAX5* was unaffected in this model, demonstrating a similar haploinsufficiency mode of action. In a similar mouse model combining constitutively active STAT5 with reversible RNAi-mediated silencing of PAX5, it was found that

even brief restoration of PAX5 expression in leukemia cells causes rapid cell cycle exit and disables their leukemia-initiating capacity [118]. Phenotypic and transcriptomic analysis demonstrated that this differentiation response closely mimics the transition of large cycling pre-B cells to small resting pre-B cells found in normal hematopoiesis [118]. In the same study also the human B-ALL cell lines REH, 697 and NALM-6 were found to be addicted to *PAX5* hypomorphism. Hence, these findings not only causally link loss of PAX5 with B-ALL initiation but demonstrate that reduced PAX5 levels are also relevant for tumor maintenance.

4. Conclusions

Taken together, the data demonstrate that inappropriate PAX protein activity is involved in tumorigenesis of different malignancies. The described mutations in PAX proteins are involved in blocking differentiation of the affected cells and keeping them in a proliferative state. It has been suggested that the most permissive state for pro-tumorigenic effects of mutations in a cell lineage is the transit-amplifying/progenitor stage [121]. At this stage cells divide rapidly, which allows for their exponential expansion. Deregulation of PAX protein activity might be causally involved in the block of the exit from such a stage in cancer cells.

Both loss of function and gain of function mechanisms have been identified and lead to the following model (Fig. 1): PAX proteins with a potential for oncogenic behavior upon activation by mutation are normally active in the proliferative state of a cell lineage and have to be downregulated before differentiation of the cells proceeds. Prime examples for this group are PAX3 and PAX7 in alveolar rhabdomyosarcoma. In contrast, PAX proteins with a tumor suppressor activity are necessary for the exit from the proliferative state of a cell linage and remain expressed during (part of) the differentiation process. Their reduction or loss therefore blocks cells in a progenitor state. Prime example for this group is PAX5 in the B-cell lineage and B-ALL.

The exact stage of the affected lineages at which the mutations in the PAX proteins have to appear is not clear yet. However, data from mouse models of both aRMS and B-ALL suggest that a certain plasticity in the affected cell lineages exists, even allowing reversion of cells from a relative late stage of the lineage to a more proliferative state early in the lineage upon inappropriate PAX protein activity. Whether there is a “point of no return” in the differentiation hierarchy after which these mutations are no longer tumorigenic, is not yet clear.

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